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(71) Applicant: SHENZHEN UNIVERSITY [CN/CN];

Xueyuan Ave 1066, Nanshan District, Shenzhen, Guangdong 518060 (CN).

(72) Inventors: LIU, Baohua; Rm 214, 2/F, A1 Bldg, 1066

Xueyuan Blvd, Nanshan District, Shenzhen, Guangdong 518060 (CN). SUN, Shimin; Rm 209, 2/F, A1 Bldg, 1066 Xueyuan Blvd, Nanshan District, Shenzhen, Guangdong 518060 (CN).

(74) Agent: PEKSUNG INTELLECTUAL PROPERTY

LTD.; No. 63201, Floor 1, Building 16, No.1 Zhongguancun Street South, Haidian District, Beijing 100873 (CN).

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(54) Title: USE OF ENDOTHELIAL PROGENITOR CELLS (EPCS) IN REJUVENATING MICROVASCULATURE, PREVENTING AGING AND TREATING AGE-RELATED DISEASES

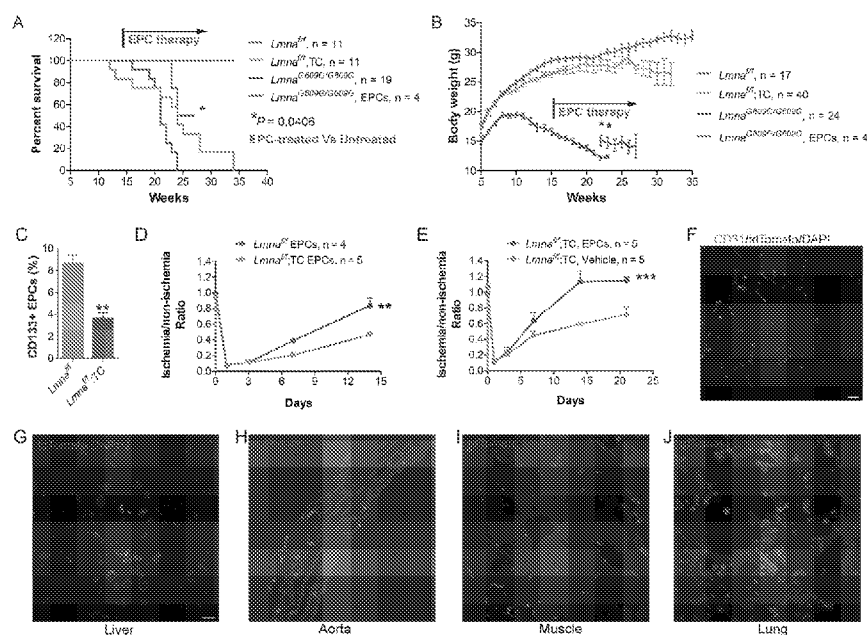


Figure 6

(57) Abstract: Disclosed are endothelial progenitor cells (EPCs), their role in preventing aging, extending lifespan and treating age-related diseases, and in particular, the use of endothelial progenitor cells in clinical progeria treatment.

Use of endothelial progenitor cells (EPCs) in rejuvenating the microvasculature, preventing aging and treating age-related diseases

Technical Field

The present invention relates to endothelial progenitor cells (EPCs) and their role in preventing aging, extending lifespan and treating age-related diseases. In particular, the present invention relates to use of endothelial progenitor cells in clinical progeria treatment.

Background

Aging represents the largest risk factor for many age-related diseases, as exemplified by vascular dysfunction and cardiovascular diseases (CVDs) (Le Couteur and Lakatta, 2010). The blood vessel consists of the tunica intima (composed of endothelial cells; ECs), the tunica media (composed of vascular smooth muscle cells; VSMCs) and the tunica adventitia (consisting of connective tissue) (Tian and Li, 2014). The endothelium separates the vessel wall from the blood flow and has an irreplaceable role in regulating vascular tone and homeostasis (Brandes et al., 2005; Hadi et al., 2005). Age-related functional decline in ECs and VSMCs is a main cause of CVDs (Brandes et al., 2005; Ghebre et al., 2016; Tao et al., 2004). ECs secrete various vasodilators and vasoconstrictors that act on VSMCs and induce blood-vessel contraction and relaxation (Ignarro et al., 2001). For instance, nitric oxide (NO) is synthesized from L-arginine by endothelial NO synthase (eNOS) in ECs and is released on VSMCs to induce blood-vessel relaxation (Cheang et al., 2014). When ECs become senescent or dysfunctional, vasoconstrictive, pro-coagulative and pro-inflammatory cytokines are released; this effect reduces NO bioavailability and in turn increases vascular intimal permeability and EC migration (Li et al., 2017). Despite advances in the understanding of the mechanisms of endothelial dysfunction, it is unclear whether it directly triggers organismal aging.

Accumulating data suggest that the mechanisms underlying normal aging are similar to those governing Hutchinson-Gilford progeria syndrome (HGPS) – a premature aging syndrome in which affected patients typically succumb to CVDs (Cao et al.,

2011; De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003; Liu et al., 2012; McClintock et al., 2007; Scaffidi and Misteli, 2006). HGPS is predominantly caused by an a c. 1824 C > T, p. G608G mutation in *LMNA* gene, which activates an alternate splicing event and generates a 50-amino-acid-truncated form of lamin A, referred to as progerin (Scaffidi and Misteli, 2006). The murine *Lmna*^{G609G}, which is equivalent to *LMNA*^{G608G} in humans, causes aging phenotypes resembling HGPS (Osorio et al., 2011). It has been shown that progerin targets SMCs and causes blood vessel calcification and atherosclerosis (Liu et al., 2011; Liu et al., 2013; McClintock et al., 2006; Ragnauth et al., 2010; Varga et al., 2006; Zhang et al., 2011). Recent work by two groups showed that SMC-specific progerin knock-in mice are healthy and have a normal lifespan, but suffer from blood-vessel calcification, atherosclerosis and shortened lifespan when crossed to *Apoe*^{-/-} mice (Hamczyk et al., 2018; Kim et al., 2018). In contrast to SMCs, the contributing roles of the vascular endothelium (VE) to systemic/organismal aging are elusive.

Endothelial progenitor cells (EPCs) mainly exist in the bone marrow (Hill et al., 2003; Williamson et al., 2012). Upon VE injury, cytokines and growth factors, such as VEGF, SDF-1, G-CSF and estrogen, mobilize EPCs to the peripheral circulation. The EPCs then seed at the injury site and promote repair via neovascularization (Ghebre et al., 2016; Hill et al., 2003). An age-related decline in the number and function of EPCs is a main reason for decreased VE repair capacity (Dantas et al., 2012; Moriya and Minamino, 2017; Williamson et al., 2012). Progeria models exhibit depleted stem cells, including mesenchymal stem cells (MSCs), epithelial stem cells, muscular stem cells and hematopoietic stem cells (HSCs) (Espada et al., 2008; Liu et al., 2011; Scaffidi and Misteli, 2008; Song et al., 2013). Questions remain as to whether EPCs also decline in progeria and if so, whether this decline causally accelerates aging. To address these issues, we generated a conditional progerin (*Lmna*^{G609G}) knock-in (KI) model, i.e. *Lmna*^{ff} mice. In combination with *E2A-Cre* and *Tie2-Cre* mice, we aimed to investigate the roles of the VE dysfunction and the EPCs to systemic aging.

Summary of the Invention

Vascular dysfunction is one of the typical characteristics of aging, but its contributing

roles to systemic aging is lacking experimental evidence. Accumulating data suggest that mechanisms underlying aging are similar to those governing Hutchinson-Gilford progeria syndrome (HGPS), a premature aging syndrome in which affected patients typically succumb to cardiovascular diseases (CVDs). Here, we generated a knock-in mouse model with the causative HGPS *Lmna*^{G609G} mutation. Using the *Lmna*^{ff} and *Tie2-Cre* mice, we showed that endothelial-specific dysfunction compromises the microvasculature and neovascularization and accelerates aging in multiple tissues/organs. Most importantly, endothelial-specific dysfunction shortens lifespan in *Lmna*^{ff};TC mice. Mechanistically, single-cell transcriptomic analysis of murine lung endothelial cells (MLECs) revealed a significant up-regulation of genes that regulate inflammation including *Il6*, *Il8*, *Il15*, *Cxcl1* and *Il1α* etc. Further determined by FACS analysis and neovascularization assay, we observed that the number and function of EPCs in the bone marrow decline in *Lmna*^{ff};TC mice compared to *Lmna*^{ff} control mice. Replenishing wild-type EPCs rejuvenates neovascularization capacity, ameliorates aging features and extends lifespan in progeria mice. These data reveal that endothelial dysfunction triggers systemic aging and highlight EPC therapy as a potential anti-aging strategy and clinical progeria treatment.

In one aspect, the present invention provides use of endothelial progenitor cells (EPCs) in the manufacture of a medicament for rejuvenating neovascularization capacity, ameliorating aging features, preventing aging, extending lifespan, and/or treating progeria and/or age-related diseases. Preferably, the age-related diseases are cardiovascular diseases and/or osteoporosis. More preferably, the cardiovascular diseases are atherosclerosis and/or heart failure.

In another aspect, the present invention provides a method for rejuvenating neovascularization capacity, ameliorating aging features, preventing aging, extending lifespan, and/or treating progeria and/or age-related diseases, comprising administering a pharmaceutically effective amount of EPCs to a subject in need thereof. Preferably, the age-related diseases are cardiovascular diseases and/or osteoporosis. More preferably, the cardiovascular diseases are atherosclerosis and/or

heart failure.

Description of the Figures

Figure 1 shows single-cell transcriptomic profiles of CD31⁺ MLECs.

- (A) Purity analysis of sorted CD31⁺ MLECs by FACS.
- (B) t-SNE projection of CD31⁺ cells revealed four clusters: endothelial cells (ECs), B lymphocytes (B-like), T lymphocytes (T-like) and Macrophages (Mφ-like).
- (C) Marker gene expression in the four clusters: ECs (*Cd31*, *Cd34*, *Cdh5*), B-like (*Ly6d*, *Cd22*, *Cd81*), T-like (*Cd3d*, *Cd3e*, *Cd28*) and Mφ-like (*Cd14*, *Cd68*, *Cd282*).
- (D) Heatmap showing marker gene expression levels in E2A and Flox mice.

Figure 2 shows that single-cell transcriptomic analysis indicates an inflammatory response and cardiac dysfunction in progeroid ECs.

- (A) t-SNE projection of *Lmna*^{G609G/G609G} (G609G) and *Lmna*^{ff} (Flox) CD31⁺ MLECs, according to transcriptomic data.
- (B-D) GO and KEGG pathway enrichment of differentially expressed genes between G609G and Flox cells. *Lmna*^{G609G/G609G} MLECs show enrichment in genes that regulate the inflammatory response (C) and genes related to heart dysfunction (D).
- (E) Quantitative PCR analysis of altered genes observed in (C) and (D) in human umbilical vein endothelial cells (HUVECs) with ectopic expression of progerin or wild type *LMNA*. Data represent the means ± s.e.m. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's t test).

Figure 3 shows endothelial-specific dysfunction in progeria mice.

- (A, B) H&E staining of thoracic aorta sections from (A) *Lmna*^{ff},TC and (B) *Lmna*^{G609G/G609G} and *Lmna*^{ff} control mice showing intima-media thickening. Scale bar, 20 μm.
- (C) Acetylcholine (ACh)-induced thoracic aorta vasodilation in *Lmna*^{ff},TC and *Lmna*^{ff} control mice. ***P* < 0.01.
- (D) ACh-induced thoracic aorta vasodilation in *Lmna*^{G609G/G609G} and control mice. ***P* < 0.01.

(E) Sodium nitroprusside (SNP)-induced thoracic aorta vasodilation in *Lmna*^{G609G/G609G} and control mice.

(E) eNOS level in thoracic aorta sections from *Lmna*^{ff};TC and control mice. Scale bar, 20 μ m.

All data represent the means $\pm$ s.e.m. P values were calculated by Student's *t* test.

Figure 4 shows reduced capillary density and defective neovascularization.

(A) Immunofluorescence staining (left) and quantification (right) of CD31⁺ gastrocnemius muscle in *Lmna*^{ff};TC and *Lmna*^{ff} mice. Scale bar, 50 μ m.

(B) CD31 immunofluorescent staining in *Lmna*^{ff};TC and *Lmna*^{ff} liver. Scale bar, 50 μ m.

(C) Representative microcirculation images (left) and quantification of blood flow recovery (right) following hind limb ischemia in *Lmna*^{ff};TC and *Lmna*^{ff} mice.

(D) Representative transverse sections and quantification of CD31⁺ gastrocnemius muscle 14 days after femoral artery ligation. Scale bar, 50 μ m.

All data represent the means $\pm$ s.e.m. P values were calculated by Student's *t* test.

Figure 5 shows systemic aging phenotypes in *Lmna*^{ff};TC mice.

(A-C) Masson trichrome staining showing an atheromatous plaque in the aorta (A), smooth muscle cell loss (B) and cardiac fibrosis (C) in *Lmna*^{ff};TC mice. Scale bar, 20 μ m.

(D) Heart weight and echocardiographic parameters, including heart rate, cardiac output, left ventricular (LV) ejection fraction and LV ejection shortening.

(E) Decreased running endurance in *Lmna*^{ff};TC mice.

(F) Micro-CT analysis showing a decrease in trabecular bone volume/tissue volume (BV/TV), trabecular number (Tb.N) and trabecular thickness (Tb.Th), and an increase in trabecular separation (Tb.Sp) in *Lmna*^{ff};TC mice.

All data represent the means $\pm$ s.e.m. P values were calculated by Student's *t* test.

Figure 6 shows that EPCs rejuvenate the microvasculature and extend lifespan in progeria mice.

- (A) Lifespan of EPC-treated and untreated *Lmna*^{G609G/G609G} mice, *Lmna*^{ff};TC and *Lmna*^{ff} mice.
- (B) Body weight of EPC-treated and untreated *Lmna*^{G609G/G609G} mice, *Lmna*^{ff};TC and *Lmna*^{ff} mice. **P* < 0.05.
- (C) Percent CD133⁺ EPCs in *Lmna*^{ff};TC and *Lmna*^{ff} mice.
- (D) Neovascularization assay of CD133⁺ EPCs derived from *Lmna*^{ff};TC and *Lmna*^{ff} mice in mice with hind limb ischemia.
- (E) EPCs from rosa26-rainbow mice rescue hind limb ischemia in *Lmna*^{ff};TC mice.
- (F) Representative immunofluorescence image showing ECs that have differentiated from rosa26-rainbow EPCs. Scale bar, 15 μ m.
- (G-H) Representative immunofluorescence images of the liver (G), aorta (H), muscle (I) and lung (J) of *Lmna*^{ff};TC mice after EPC therapy, showing ECs that have differentiated from rosa26-rainbow EPCs. Scale bar, 15 μ m.

All data represent the means $\pm$ s.e.m. P values were calculated by Student's *t* test.

Figure 7 shows generation of *Lmna*^{ff} mice and phenotypic analysis of *Lmna*^{G609G/G609G} mice.

- (A) Schematic illustration of knock-in strategy of *Lmna*^{ff} mice harboring *Lmna*^{G609G} mutation (*Lmna* 1827C>T)
- (B) Representative photo of *Lmna*^{G609G/G609G} mice and *Lmna*^{ff} control mice.
- (C) Representative immunoblot showing Lamin A, Progerin and Lamin A expression in *Lmna*^{G609G/+}, *Lmna*^{G609G/G609G} and *Lmna*^{+/+} control mice.
- (D) Lifespan determination of *Lmna*^{G609G/+}, *Lmna*^{G609G/G609G} and *Lmna*^{+/+} mice.

Figure 8 shows single cell transcriptomic analysis of CD31⁺ MLECs.

- (A) *p21*^{Cip/Waf1} mRNA levels in *Lmna*^{G609G/G609G} (G609G) and *Lmna*^{ff} (Flox) CD31⁺ MLECs. *p21*^{Cip/Waf1} was elevated specifically in ECs and MΦ-like cells isolated from G609G mice.
- (B) *Cd45* and *Tie2* levels in G609G and Flox CD31⁺ MLECs. *Cd45* expression was lacking in ECs and *Tie2* expression was EC-specific.

Figure 9 shows VE-specific progerin expression.

(A-B) Progerin and CD31 expression was detected by immunofluorescence staining in aorta (A) and muscle (B) tissue of *Lmna*^{ff};TC and *Lmna*^{ff} mice.

Figure 10 shows vasodilation analysis of *Lmna*^{G609G/+} mice.

Acetylcholine (ACh)-induced (left) and sodium nitroprusside (SNP)-induced (right) vasodilation in *Lmna*^{G609G/+} and *Lmna*^{+/+} control mice.

Figure 11 shows the expression of atherosclerosis-associated and osteoporosis-associated genes in MLEC transcriptomes.

Figure 12 shows CD133⁺ Endothelial progenitor cells labeled with Dil-acLDL and UEA. The nuclei were counterstained with DAPI. Scale bar, 50 μ m.

Figure 13 shows comparison of expression levels of genes that are associated with atherosclerosis, arthritis, heart failure, osteoporosis and amyotrophy in different clusters of cells recovered from the single-cell RNA sequencing.

Particular Embodiments

The present invention provides use of endothelial progenitor cells (EPCs) in the manufacture of a medicament for rejuvenating neovascularization capacity, ameliorating aging features, preventing aging, extending lifespan, and/or treating progeria and/or age-related diseases, more preferably atherosclerosis and/or heart failure.

In a specific embodiment, the EPCs are CD133⁺ EPCs.

In a specific embodiment, the age-related diseases are characterized by vascular endothelium (VE) dysfunction.

In particular, the VE dysfunction includes a loss of endothelial cells, reduced capillary density and defective neovascularization capacity.

More particularly, the VE dysfunction is caused by progerin.

The present invention also provides a method for rejuvenating neovascularization capacity, ameliorating aging features, preventing aging, extending lifespan, and/or

treating progeria and/or age-related diseases, comprising administering a pharmaceutically effective amount of EPCs to a subject in need thereof; preferably, the age-related diseases are cardiovascular diseases and/or osteoporosis, more preferably atherosclerosis and/or heart failure.

In a specific embodiment, the EPCs are CD133⁺ EPCs.

In a specific embodiment, the age-related diseases are characterized by vascular endothelium (VE) dysfunction.

In particular, the VE dysfunction includes a loss of endothelial cells, reduced capillary density and defective neovascularization capacity.

More particularly, the VE dysfunction is caused by progerin.

The present invention will be further demonstrated by the following experimental procedures and examples, which are used only for illustration purpose, but not limiting the scope of the present invention.

Experimental procedures

Animals

Lmna^{ff} alleles (*Lmna*^{G609G} flanked by 2 loxP sites) were generated accordingly. The 5' and 3' homology arms were amplified from BAC clones RP23-21K15 and RP23-174J9, respectively. The G609G (GGC to GGT) mutation was introduced into exon 11 in the 3' homology arm. C57BL/6 embryonic stem cells were used for gene targeting. To obtain ubiquitous progerin expression (*Lmna*^{G609G/G609G}), *Lmna*^{ff} mice were bred with *E2A-Cre* mice. To obtain VE-specific progerin expression, *Lmna*^{ff} mice were bred with *Tie2-cre* mice. Mice were purchased from Cyagen Biosciences Inc., China, housed and handled in accordance with protocols approved by the Committee on the Use of Live Animals in Teaching and Research of Shenzhen University, China.

Hind limb ischemia

Four months old male mice were anesthetized with 4% chloral hydrate (0.20 ml/20 g) by intraperitoneal injection. Hind limb ischemia was performed by unilateral femoral artery ligation and excision, as previously described (Limboureg et al., 2009). In brief, the neurovascular pedicle was visualized under a light microscope following a 1-cm

incision in the skin of the left hind limb. Ligations were made in the left femoral artery proximal to the superficial epigastric artery branch and anterior to the saphenous artery. Then, the femoral artery and the attached branches between ligations were excised. The skin was closed using a 4-0 suture line and erythromycin ointment was applied to prevent wound infection after surgery. Recovery of the blood flow was evaluated before and after surgery using a dynamic microcirculation imaging system (Teksqray, Shenzhen, China). Relative blood flow recovery is expressed as the ischemia to non-ischemia ratio. At least three mice were included in each experimental group.

Cell culture

HEK293 cells and human umbilical vein endothelial cells (HUVECs) were purchased from ATCC. HEK293 cells were cultured in Gibco[®] DMEM (Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C, 5% CO₂. HUVECs were cultured in Gibco[®] M199 (Life Technologies, USA) supplemented with 15% FBS, 50 µg/ml endothelial cell growth supplement (ECGS) and 100 µg/ml heparin at 37°C, 5% CO₂. All cell lines used were authenticated by short tandem repeat (STR) profile analysis and were mycoplasma free.

RNA isolation and quantitative PCR (Q-PCR) analysis

Total RNA was extracted from cells or mouse tissues using Trizol[®] reagent RNAiso Plus (Takara, Japan) and transcribed into cDNA using 5 × Primescript RT Master Mix (Takara, Japan), following the manufacturer's instructions. The mRNA levels were determined by quantitative PCR with SYBR Premix Ex Taq II (Takara, Japan) detected on a CFX Connected Real-Time PCR Detection System (Bio-Rad). All primer sequences are listed in Table 1.

Table 1

Targets (Homo)	Forward	Reverse
<i>hIL15</i>	GCAATGTTCCATCATGTTCC	GCCTCCTACAATACAATACGA
<i>hCXCL1</i>	CTGAACAGTGACAAATCCAA	GGGGTTGACATTTCAAAAAGAA
<i>hCCL2</i>	TGAGACTAACCCAGAAACATC	CTTGAAGATCACAGCTTCTTT

<i>IL1β</i>	CATTGCTCAAGTGTCTGAAG	TTCATCTGTTTAGGGCCATC
<i>CXCL2</i>	CCAACCATGCATAAAAGGGG	GGGGCGCTCCTGCTG
<i>PTGIS</i>	AGCTTCCACATTACAGCCCC	AGGAGAAGTCGAGGAGACCC
<i>TGFβ2</i>	CGAAACTGTCTGCCCAGTTG	TGTAGAAAGTGGGCGGGATG
<i>CXCL14</i>	CTAAGATGACCATGCGCCCT	AATGCGGCATATACTGGGGG
<i>SERPINE1</i>	GCAAGGCACCTCTGAGAACT	GGGTGAGAAAACCACGTTGC
<i>Progerin</i>	GTTGAGGACGACGAGGATGAG	CAGTTCTGGGGGCTCTGGGCTC
<i>hIL1A</i>	TGAGTCAGCAAAGAAGTCAA	GATTGGCTTAAACTCAACCG
<i>IL6</i>	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
<i>β-actin</i>	AGAGCTAGCTGCCTGAC	GGATGCCACAGGACTCCA

Protein extraction and Western blotting

For protein extraction, cells were suspended in SDS lysis buffer and boiled. Then, the lysate was centrifuged at 12,000 $\times$ g for 2 min and the supernatant was collected. For Western blotting, protein samples were separated on SDS-polyacrylamide gels, transferred to PVDF membranes (Millipore, USA), blocked with 5% non-fat milk and incubated with the relevant antibodies. Images were acquired on a Bio-Rad system. All antibodies are listed in Table 2.

Table 2

Antibodies	M/R/Rat		Vendor	Dilution
VEGFR2	rabbit	ab2349	abcam	1:100
CD31	rabbit	ab28364	abcam	1:100
SMA	rabbit	ab5694	abcam	1:500
CD31	rat	ab7388	abcam	1:50
progerin	mouse	ab66587	abcam	1:50
LaminA/C	mouse	ab8984	abcam	1:100
Enos	mouse	ab76198	abcam	1:100
LaminA	mouse	sc-71488	Santa Cruz	1:100
progerin	mouse	ab-81611	Santa Cruz	1:50
LaminA/C	rabbit	sc-20681	Santa Cruz	1:100
PE-CD31	rat	553373	BD pharmingen	1:100
PE-CD34	rat	551387	BD pharmingen	1:250
PE-Flk-1	rat	555308	BD pharmingen	1:250
PE-CD117	rat	553355	BD pharmingen	1:250
PE-IgG2b k Isotype	rat	555848	BD pharmingen	1:100

control				
Alexa Fluor 488 -IgG2a k Isotype control	rat	557676	BD pharmingen	1:250
Purified Anti-mouse CD16/CD32	rat	553141	BD pharmingen	1:250
APC-CD133	mouse	141208	Biolegend	1:250
APC-IgG2a k Isotype control	rat	400511	Biolegend	1:250
Alexa Fluor 488-Flk-1	mouse	121908	Biolegend	1:250
Alexa Fluor 488- IgG2a k Isotype control	rat	400525	Biolegend	1:250
prominin-1-biotin(CD133)	mouse	130-111-353	Miltenyi Biotec	1:100
CD34-biotin	mouse	130-105-830	Miltenyi Biotec	1:100
Flk-1-microBeads-Kit	mouse	130-097-346	Miltenyi Biotec	
MACS separation buffer		130-091-221	Miltenyi Biotec	
Anti-biotin-microBeads		130-090-485	Miltenyi Biotec	1:4
Dil-ac-LDL		L-3484	invitrogen	1:100
FITC-UEA-1		L9006-1MG	SIGMA	1:100

Immunofluorescence staining

Aorta, skeletal muscle and liver tissues were collected from *Lmna*^{G609G/G609G}, *Lmna*^{+/+}, *Lmna*^{ff}, TC and *Lmna*^{ff} mice. Frozen sections were prepared and fixed in 4% PFA, permeabilized with 0.3% Triton X-100, blocked with 5% BSA and 1% goat serum, and then incubated with primary antibodies at room temperature for 2 h or at 4°C overnight. After three washes with PBST, the sections were incubated with secondary antibodies for 1 h at room temperature and then stained with DAPI anti-fade mounting medium. Images were captured under a Zeiss LSM880 confocal microscope. All antibodies are listed in Table 2.

Masson trichrome staining

Paraffin-embedded sections of PFA-fixed tissues were dewaxed and hydrated. Staining was then performed using a Masson trichrome staining kit (Beyotime, China). In brief, the sections were dipped in Bouin buffer for 2 h at 37°C, and then successively stained with Celestite blue staining solution, Hematoxylin staining solution, Ponceau's staining solution and Aniline blue solution for 3 minutes. After dehydrating with ethyl alcohol three times, the sections were mounted with Neutral

Balsam Mounting Medium (BBI Life Science, China). Images were captured under a Zeiss LSM880 confocal microscope.

Fluorescence activated cell sorting (FACS)

Mice were sacrificed by decapitation. The lungs were then collected, cut into small pieces and then digested with collagenase I (200 U/ml) and neutral protease (0.565 mg/ml) for 1 h at 37°C. The isolated cells were incubated with PE-conjugated anti CD31 antibody for 1 h at 4°C and then 7-AAD (1:100) for 5 min. CD31-positive and 7-AAD-negative cells were sorted on a flow cytometer (BD biosciences, USA).

Myography

Four months old male mice were anesthetized with 4% chloral hydrate by intraperitoneal injection. Thoracic aortas were collected, rinsed in ice-cold Krebs solution and cut into 2 mm-length rings. Each aorta ring was bathed in 5 ml oxygenated (95% O₂ and 5% CO₂) Krebs solution at 37°C for 30 min in a myograph chamber (620M, Danish Myo Technology). Each ring was stretched in a stepwise fashion to the optimal resting tension (thoracic aortas to ~9 mN) and equilibrated for 30 min. Then, 100 mM K⁺ Krebs solution was added to the chambers to elicit a reference contraction and then washed out with Krebs solution at 37°C until achieved a baseline. Vasodilatation induced by acetylcholine (Ach) or sodium nitroprusside (SNP) (1 nM to 100 μM) was recorded in 5-HT (2 μM) contracted rings. Data are represented as a percentage of force reduction and the peak of K⁺-induced contraction. At least three mice were included in each experimental group.

Mice/Human Cytokine Antibody Array

A cytokine assay for mice or human samples (RayBio[®]) was performed according to the manufacturer's instructions. Briefly, membranes were incubated in blocking buffer for 30 min at room temperature. The samples prepared from serum or cell lysates were added to each membrane and incubated for 4 h at room temperature. After three washes with buffer 1 and two washes with buffer 2, the membranes were reacted with a biotinylated antibody cocktail at 4°C overnight. After incubation with 1000 ×

HRP-Streptavidin for 2 h, the membranes were again washed three times with buffer 1 and two times with buffer 2 and then visualized using a Bio-Rad detection system. At least three mice were included in each experimental group.

Echocardiography

7-8 months old male mice were anesthetized by isoflurane gas inhalation and then subjected to transthoracic echocardiography (IU22, Philips). Parameters, including heart rate, cardiac output, left ventricular posterior wall dimension (LVPWD), left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic diameter (LVESD), LV ejection fraction and LV fractional shortening were acquired. At least three mice were included in each experimental group.

Bone density analysis

7-8 months old male mice were sacrificed by decapitation. The thigh bone was fixed in 4% PFA at 4°C overnight. The relevant data were collected by micro-CT (Scanco Medical, μ CT100). At least three mice were included in each experimental group.

Endurance running test

A Rota-Rod Treadmill (YLS-4C, Jinan Yiyan Scientific Research Company, China) was used to monitor fatigue resistance. Briefly, mice were placed on the rotating lane and the speed of the rotations gradually increased to 40 r/min. When the mice were exhausted, they were safely dropped from the rotating lane and the latency to fall was recorded. At least three mice were included in each experimental group.

10 × Genomics single-cell-RNA-sequencing

CD31⁺ cells isolated from murine lung by FACS (>90% viability) were used for single-cell RNA sequencing. A sequence library was built according to the Chromium Single Cell Instrument library protocol (Neal et al., 2018). Briefly, single-cell RNAs were barcoded and reverse-transcribed using Chromium™ Single Cell 3' Reagent Kits v2, then fragmented and amplified to generate cDNAs. The cDNAs were quantified using an Agilent Bioanalyzer 2100 DNA Chip, and the library was

sequenced using an Illumina Hiseq PE150 with ~10-30M raw data assigned for each cell. The reads were mapped to the mouse mm9 genome and analyzed using STAR: >90% reads mapped confidently to genomic regions and >50% mapped to exonic regions. Cell Ranger 2.1.0 was employed to align reads, generate feature-barcode matrices and perform clustering and gene expression analysis. >80,000 mean reads and 900 median genes per cell were obtained. The UMI (unique molecular identifier) counts were used to quantify the gene expression levels and the t-SNE algorithm was used for dimensionality reduction. The cell population was then clustered by k-means clustering ($k = 4$). The Log2FoldChange was the ratio of gene expression of one cluster to that of all other cells. The p-value was calculated using the negative binomial test and the false discovery rate was determined by Benjamini-Hochberg procedure. GO and KEGG enrichment analysis were performed in DAVID version 6.8.(Huang da et al., 2009)

Isolation of CD133⁺ progenitor cells

3 months old male mice were sacrificed by decapitation. The femora and tibiae were separated and placed in a 0.5 ml micro-centrifuge tube which had a hole drilled in the bottom. A 1.5 ml micro-centrifuge tube was used to nest the 0.5 ml tube and the pair of tubes was centrifuged at 10,000×g for 15 sec. The bone marrow was suspended in 1 ml red blood cell lysis buffer at room temperature for 5 min, and the suspension was strained successively through a 75-μm and then 40-μm cell strainer (FALCON®, USA). After centrifugation at 300×g at 4°C for 5 min, the cells were suspended in 500 μl MACS buffer and incubated with 5 μl anti-CD133 antibody (Miltenyi Biotec, Germany) for 10 min. After incubating with 20 μl beads (Miltenyi Biotec, Germany) in 80 μl MACS buffer, CD133⁺ progenitor cells were obtained by magnetic selection. At least three mice were included in each experimental group.

Statistical analysis

A two-tailed Student's t-test was used to determine statistical significance. All data are presented as the means ± s.d. or means ± s.e.m. as indicated, and a p value < 0.05 was considered statistically significant.

Example 1

Single-cell transcriptomic analysis reveals four predominant cell clusters in CD31⁺ murine lung endothelial cells (MLECs)

An outstanding question in the field of aging is whether endothelial dysfunction causally triggers systemic aging. The heterogeneity of vascular cells and their close communication with the blood stream, however, renders it difficult to understand the primary function of the VE. The murine *Lmna*^{G609G} mutation, which is equivalent to human *LMNA*^{G608G}, causes aging phenotypes in various tissues resembling HGPS. To examine the contributing roles of the VE to systemic aging, we generated a mouse model of conditional progerin knock-in, in which the *Lmna*^{G609G} mutation was flanked with loxP sites, i.e. *Lmna*^{ff} mice (Figure 7A). The *Lmna*^{ff} mice were crossed to *E2A-Cre* mice, in which Cre recombinase is ubiquitously expressed including germ cells, to generate *Lmna*^{G609G/G609G} mice. Progerin was ubiquitously expressed in these *Lmna*^{G609G/G609G} mice, which recapitulated many progeroid features found in HGPS, including growth retardation and shortened lifespan etc. (Figure 7B-D).

To understand the primary alterations in the VE, we isolated CD31⁺ MLECs (Longchamp et al., 2018) from three pairs of *Lmna*^{G609G/G609G} (G609G) and *Lmna*^{ff} (Flox) control mice by FACS (Figure 1A) and performed 10× Genomics single-cell RNA sequencing. We recovered 6,004 cells (4,137 from G609G and 1,867 from Flox mice) and used the k means clustering algorithm to cluster the cells into four groups (Figure 1B). As expected, one group exhibited high *Cd31*, *Cd34* and *Cdh5* expression, and thus largely represented MLECs. The other three groups, co-purified with CD31⁺ MLECs by FACS, showed relatively lower *Cd31* expression (>10-fold lower than MLECs) but high *Cd45* expression (Figure 8). Further analysis revealed that these clusters most likely contained B lymphocytes (B-like), with high *Cd22*, *Cd81* and *Ly6d* expression; T lymphocytes (T-like) with high *Cd3d*, *Cd3e* and *Cd28* expression; and Macrophages (Mφ-like) with high *Cd14*, *Cd68* and *Cd282* expression (Figure 1C). Most of the marker-gene expression levels were comparable between G609G and Flox mice, except for *Cd34* and *Icam1*, which were significantly elevated in G609G ECs, and *Cd14* and *Vcam1*, which were increased in G609G Mφ-like cells (Figure

1D). Of note, *Icam1* and *Vcam1* are among the most conserved markers of endothelial senescence and atherosclerosis. Thus, we established a *Lmna*^{ff} conditional progerin KI mouse model and revealed a unique EC population for mechanistic study.

Example 2

Progeroid ECs exhibit a systemic inflammatory response

Of the four clusters of CD31⁺ MLECs, ECs and Mφ-like cells showed high levels of *p21*^{Cip1/Waf1} (Figure 8A), a typical senescence marker. This finding suggests that these cells are the main target of progerin in the context of aging. Interestingly, a previous study reported that Mφ-specific progerin, achieved by crossing *Lmna*^{ff} to *Lyz-Cre* mice, caused minimal aging phenotypes (Hamczyk et al., 2018), implicating that Mφ might have only a minor role in organismal aging. We thus focused on ECs for further analysis. We recovered 899 and 445 ECs from E2A and Flox mice, respectively (Figure 2A). Genes with >1.5-fold change in expression between these mice were chosen for GO and KEGG analysis. We observed a significant enrichment in the pathways that regulate chemotaxis, immune responses in Malaria and Chagas diseases, inflammatory bowel disease and rheumatoid arthritis and pathways essential for cardiac function (Figure 2B-D). To confirm this observation, and to exclude paracrine effects from other cell types, we over-expressed progerin in human umbilical vein endothelial cells (HUVECs) and analyzed the representative genes by quantitative PCR. The majority of the examined genes, including *IL6*, *IL8*, *IL15*, *CXCL1* and *IL1α* were significantly up-regulated upon ectopic progerin expression (Figure 2E). Together, these data suggest that progerin might cause an inflammatory response in ECs, which leads to systemic aging in various organs.

Example 3

VE dysfunction promotes vasodilation defects in progeria mice

Our single-cell transcriptomic analysis in MLECs and quantitative PCR in HUVECs suggest that the VE have essential roles in systemic aging. To confirm these findings, we crossed the *Lmna*^{ff} mice to a *Tie2-Cre* line, in which Cre recombinase expression is driven by the promoter/enhancer of endothelial-specific *Tie2* gene (Kisanuki et al.,

2001), to generate *Lmna*^{ff};TC mice. Single-cell transcriptome analysis confirmed that *Tie2* gene was mainly detected in ECs (Figure 8B). Consistently, progerin was only observed in the VE of *Lmna*^{ff};TC but not *Lmna*^{ff} control mice or other tissues (Figure 9). VE-specific progerin induced intima-media thickening in *Lmna*^{ff};TC mice, in a similar manner as *Lmna*^{G609G/G609G} mice (Figure 3A-B). We next performed a functional analysis of the VE based on acetylcholine (ACh)-regulated vasodilation. ACh-induced thoracic aorta relaxation was significantly compromised in *Lmna*^{ff};TC mice (Figure 3C). Similar defects were observed in *Lmna*^{G609G/G609G} and *Lmna*^{G609G/+} mice (Figure 3D and 10), where progerin was expressed in both ECs and SMCs (Hamczyk et al., 2018). To gain more evidence supporting VE-specific dysfunction, we examined thoracic aorta relaxation induced by sodium nitroprusside (SNP), which is a SMC-dependent vasodilator. Little difference was observed in thoracic aorta vasodilation in *Lmna*^{G609G/G609G} and *Lmna*^{G609G/+} compared to *Lmna*^{ff} control mice (Figure 3E and Figure 10), supporting that the VE dysfunction is a key contributor of vasodilation defects in progeria mice. As NO is the most potent vasodilator, we examined eNOS levels in thoracic aorta of *Lmna*^{ff};TC and *Lmna*^{ff} control mice. As expected, the level of eNOS was significantly reduced in *Lmna*^{ff};TC mice compared to *Lmna*^{ff} control mice (Figure 3F). Thus, the data confer a VE-specific dysfunction in progeria mice.

Example 4

Progeria mice show defective neovascularization following ischemia

Reduced capillary density and neovascularization capacity are both characteristics of vascular aging (Le Couteur and Lakatta, 2010). We thus examined the microvasculature in various tissues of *Lmna*^{ff};TC mice by immunofluorescence staining. We observed a significant loss in CD31⁺ ECs in *Lmna*^{ff};TC mice compared to controls (Figure 4A-B). We further examined ischemia-induced neovascularization ability in *Lmna*^{ff};TC mice following femoral artery ligation. Indeed, limb perfusion after ischemia was significantly blunted in *Lmna*^{ff};TC mice compared to controls (Figure 4C). Histological analysis confirmed that the defect in blood-flow recovery in *Lmna*^{ff};TC mice was a reflection of an impaired ability to form new blood vessels in

the ischemic region (Figure 4D). Taken together, *Lmna*^{ff};TC mice are characterized by a loss of ECs, a reduced capillary density and defective neovascularization capacity.

Example 5

Endothelial dysfunction is a causal factor of systemic aging

Our single-cell transcriptomic data implicated heart dysfunction in *Lmna*^{G609G/G609G} mice (Figure 2). We also observed a significant correlation with gene alterations associated with atherosclerosis and osteoporosis in *Lmna*^{G609G/G609G} ECs (the Online Mendelian Inheritance in Man database) (Figure 11). We thus reasoned that endothelial dysfunction might trigger systemic aging. Strikingly, atherosclerosis was prominent in *Lmna*^{ff};TC mice (Figure 5A; aorta atheromatous plaque observed in all eight examined mice), as well as severe fibrosis in the arteries and hearts (Figure 5B-C); both are typical features of aging. Moreover, the heart/body weight ratio was significantly increased in *Lmna*^{ff};TC compared to *Lmna*^{ff} control mice (Figure 5D). Echocardiography confirmed that the heart rate and cardiac output were significantly reduced in 7-8-month old *Lmna*^{ff};TC compared to *Lmna*^{ff} control mice. Both the left ventricular ejection fraction (LVEF) and fractional shortening (LVFS) were below the normal values of healthy mice, which are 54% and 28% respectively. We also found that the running endurance was largely compromised in *Lmna*^{ff};TC mice (Figure 5E), which is likely a reflection of amyotrophy and/or heart dysfunction. Finally, micro-computed tomography (micro-CT) identified a decrease in trabecular bone volume/tissue volume (BV/TV), trabecular number (Tb.N) and trabecular thickness (Tb.Th), but an increase of trabecular separation (Tb.Sp) in *Lmna*^{ff};TC mice (Figure 5F), indicative of osteoporosis, which is also a hallmark of aging (Chen et al., 2013). Together, these results implicate that endothelial dysfunction, at least in the context of progeria, acts as a causal factor of systemic aging.

Example 6

EPCs rejuvenate the microvasculature, ameliorate aging and extend lifespan

The VE-specific dysfunction not only accelerated aging in various tissues/organs, but

also shortened the median lifespan in *Lmna*^{ff};TC mice (24 weeks), to a similar extent as *Lmna*^{G609G/G609G} mice (21 weeks) (Figure 6A). While *Lmna*^{G609G/G609G} mice suffered from body-weight loss from 8 weeks of age, *Lmna*^{ff};TC mice only showed a slight drop in body weight (Figure 6B). These data suggest that body-weight loss itself is a less likely primary contributing factor to progeria than endothelial dysfunction.

CD133⁺ mononuclear cells are enriched in the bone marrow and are potential EPCs that are essential for vascular hemostasis (Ghebre et al., 2016; Hill et al., 2003). We purified CD133⁺ EPCs from *Lmna*^{ff};TC mice and *Lmna*^{ff} control mice by FACS and studied the functional relevance in VE dysfunction and aging. Here, we found that >30% of the freshly isolated EPCs were positive for low density lipoproteins (as indicated by Dil-acLDL labeling) and *Ulex europaeus* agglutinin 1 (UEA-1) (Figure 12), indicating the endothelial potential of the EPCs (Asahara et al., 1997). We then analyzed EPCs in progeria mice. The number of EPCs dropped by up to 50% in *Lmna*^{ff};TC mice compared to *Lmna*^{ff} control (Figure 6C). The neovascularization ability of *Lmna*^{ff};TC EPCs was compromised more than 30% (Figure 6D). We next asked whether EPC decline contributes to the neovascularization defect in progeria mice. On-site injection of CD133⁺ EPCs isolated from rosa26-rainbow mice (tdTomato labeled) completely restored the neovascularization defect in *Lmna*^{ff};TC mice, and histological analyses confirmed the presence of donor-derived ECs in the regenerated vasculature of *Lmna*^{ff};TC mice (Figure 6E-F).

We then asked whether EPCs have a causal role in accelerating aging and shortening lifespan in progeria mice. To this end, we injected (via the tail vein) 1×10⁶ MACS (Magnetic-activated cell sorting)-purified EPCs from rosa26-rainbow mice into *Lmna*^{G609G/G609G} mice. EPCs were administered from 15 weeks before the earliest death event in *Lmna*^{G609G/G609G} mice and repeated every week. Two of the EPC-treated mice were still alive at 27 weeks-of-age and were sacrificed for histological analysis. Donor-derived ECs were detected by fluorescence microscopy in the liver, muscle, aorta and lung (Figure 6G-J, tdTomato labeled). Capillary density (CD31⁺ gastrocnemius muscle) significantly increased from 347.2±121.5 (untreated) to 581.5±85.6 (EPC-treated). More importantly, age-related body-weight loss was significantly attenuated upon EPC therapy in *Lmna*^{G609G/G609G} mice (Figure 6B), and

the median lifespan was extended from 21 to 27 weeks (Figure 6A). A reduced systemic inflammatory response was confirmed by an antibody array detecting protein factors in the blood serum (Figure 12). Altogether, these data suggest that progerin-caused endothelial dysfunction and systemic aging are partially, if not entirely, attributable to EPC decline.

Discussion

Mounting evidence supports that endothelial dysfunction is a conspicuous marker for vascular aging and CVDs (Cui et al., 2014; de la Sierra and Larrousse, 2010; Liu et al., 2017). Whether endothelial dysfunction primarily triggers organismal aging, however, is elusive. The murine *Lmna*^{G609G} mutation, equivalent to the *LMNA*^{G608G} found in humans with HGPS, causes premature aging phenotypes in various tissues/organs, thus providing an ideal model for studying aging mechanisms at both the tissue and organismal level. Data from the *Lmna*^{G609G} model have suggested that SMCs are the primary cause of vascular diseases, such as atherosclerosis (Liu et al., 2011; Liu et al., 2013; McClintock et al., 2006; Ragnauth et al., 2010; Varga et al., 2006; Zhang et al., 2011). Interestingly, a recent study showed that the specific expression of *Lmna*^{G609G} in SMCs only causes atherosclerosis and shortens lifespan in atherosclerosis-prone apolipoprotein E-deficient (*Apoe*^{-/-}) mice (Hamczyk et al., 2018). The researchers also found that macrophage-specific *Lmna*^{G609G} knock-in mediated by *Lyz-Cre* merely affects aging and lifespan. Here, we used *Tie2-Cre* mice to generate a VE-specific *Lmna*^{G609G} model. These mice exhibited vascular dysfunction, accelerated aging and a shortened lifespan to a similar extent to the whole body *Lmna*^{G609G} model. In support of our findings, Foisner *et al.* recently reported that the VE-cadherin promoter-driven endothelial-specific expression of progerin in a transgenic line causes cardiovascular abnormalities and shortened lifespan (Osmanagic-Myers et al., 2018). The data from both our study and that of Foisner strongly suggest that, as the largest secretory organ (Brandes et al., 2005; Hadi et al., 2005), the VE is pivotal in regulating systemic aging and lifespan.

One limitation in the understanding of mechanisms of VE dysfunction is the vascular cell heterogeneity and the lack of appropriate *in vitro* system for ECs. Here, we took

advantage of single-cell RNA sequencing technique to analyze the transcriptomes of MLECs. Surprisingly, although >95% purity was achieved by FACS, MLECs isolated by CD31-immunofluorescence labeling turned out to be a mixture of cells, including ECs, T-like, B-like and M ϕ -like cells. It is unclear whether these cells are T cells, B cells and M ϕ cells that express low level of CD31 (Bantikassegn et al., 2015), or are transdifferentiated from ECs. Nevertheless, this finding suggests that one can't just purify CD31⁺ cells and pool them together for further mechanistic study, otherwise might get misleading conclusion. Indeed, we compared the expression of genes that are associated with atherosclerosis, arthritis, heart failure, osteoporosis or amyotrophy (the Online Mendelian Inheritance in Man database) between progeroid and control in all four clusters. An obvious alteration of these genes/pathway was observed mainly in ECs and M ϕ -like cells (Figure 13). As we did single-cell transcriptomic analysis in *Lmna*^{G609G/G609G} mice, it is difficult to separate cell-autonomous and paracrine effects among different cell populations. In future study, it is worth to do a similar analysis in *Lmna*^{ff},TC MLECs. The data will be useful to study the paracrine effect of ECs on the other cell populations.

The stem-cell theory of aging dictates that the number and functional decline of stem cells directly leads to defective tissue regeneration and consequently organismal aging. EPCs, MSCs and HSCs represent 3 stem-cell populations found in the bone marrow, of which the latter two have implicated clinical potential. We previously showed that the number and function of MSCs and HSCs decline in another progeria mouse model, *Zmpste24*^{-/-} mice (Liu et al., 2012); however, we didn't observe any beneficial effect when MSCs from healthy donors was transplanted into *Zmpste24*^{-/-} mice by tail vein injection (Figure S8). Consistent with the rapid decline of HSCs and MSCs, we found that the number and function of EPCs, represented by CD133⁺ mononuclear cells, significantly declined in progeria mice compared to healthy controls. Remarkably, EPC transplantation via tail vein injection improved the microvasculature, attenuated body-weight loss, and extended lifespan in progeria mice. To our knowledge, this study provides the first evidence to support the potential of stem-cell therapy in progeria treatment. We thus consider it worthwhile to optimize the conditions of this therapy to maximize the rescue effect elicited by EPCs, and to screen for chemicals

that increase the number, improve the function and promote the mobilization of EPCs. Indeed, various drugs clinically used to treat CVDs, such as statins and PPAR γ agonists, can mobilize EPCs from the bone marrow to peripheral circulation and enhance endothelial repair. Thus, further investigation is warranted to examine whether these drugs can slow down aging and promote longevity.

Collectively, we reveal that VE dysfunction is a trigger of systemic aging and is also a risk factor for age-related diseases like atherosclerosis, heart failure and osteoporosis. It suggests that many clinically used drugs and molecules that target VE might serve as good candidates in the treatment of age-related diseases other than CVDs. Likewise, the findings in EPCs implicate great potentials of stem-cell-based therapeutic strategy for progeria as well as in anti-aging applications.

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Claims

1. Use of endothelial progenitor cells (EPCs) in the manufacture of a medicament for rejuvenating neovascularization capacity, ameliorating aging features, preventing aging, extending lifespan, and/or treating progeria and/or age-related diseases.
2. Use according to claim 1, wherein the EPCs are CD133⁺ EPCs.
3. Use according to claim 1 or 2, wherein the age-related diseases are cardiovascular diseases and/or osteoporosis; preferably, the cardiovascular diseases are atherosclerosis and/or heart failure.
4. Use according to any one of claims 1-3, wherein the diseases are characterized by vascular endothelium (VE) dysfunction.
5. Use according to claim 4, wherein the VE dysfunction includes a loss of endothelial cells, reduced capillary density and defective neovascularization capacity.
6. Use according to claim 4 or 5, wherein the VE dysfunction is caused by progerin.
7. A method for rejuvenating neovascularization capacity, ameliorating aging features, preventing aging, extending lifespan, and/or treating progeria and/or age-related diseases, comprising administering a pharmaceutically effective amount of EPCs to a subject in need thereof; preferably, the age-related diseases are cardiovascular diseases and/or osteoporosis, more preferably atherosclerosis and/or heart failure.
8. The method according to claim 7, wherein the EPCs are CD133⁺ EPCs.
9. The method according to claim 7 or 8, wherein the age-related diseases are characterized by vascular endothelium (VE) dysfunction; particularly, the VE dysfunction includes a loss of endothelial cells, reduced capillary density and defective neovascularization capacity.
10. The method according to claim 9, wherein the VE dysfunction is caused by progerin.

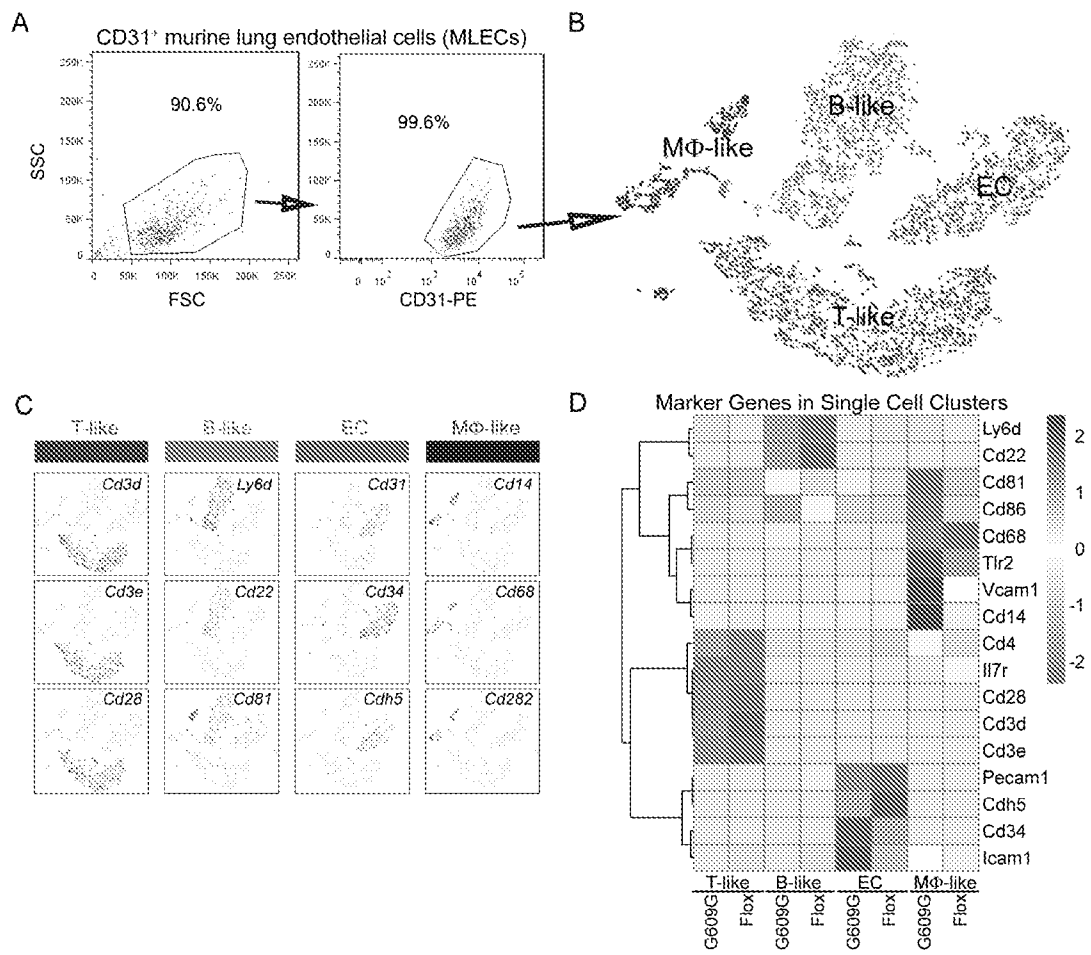
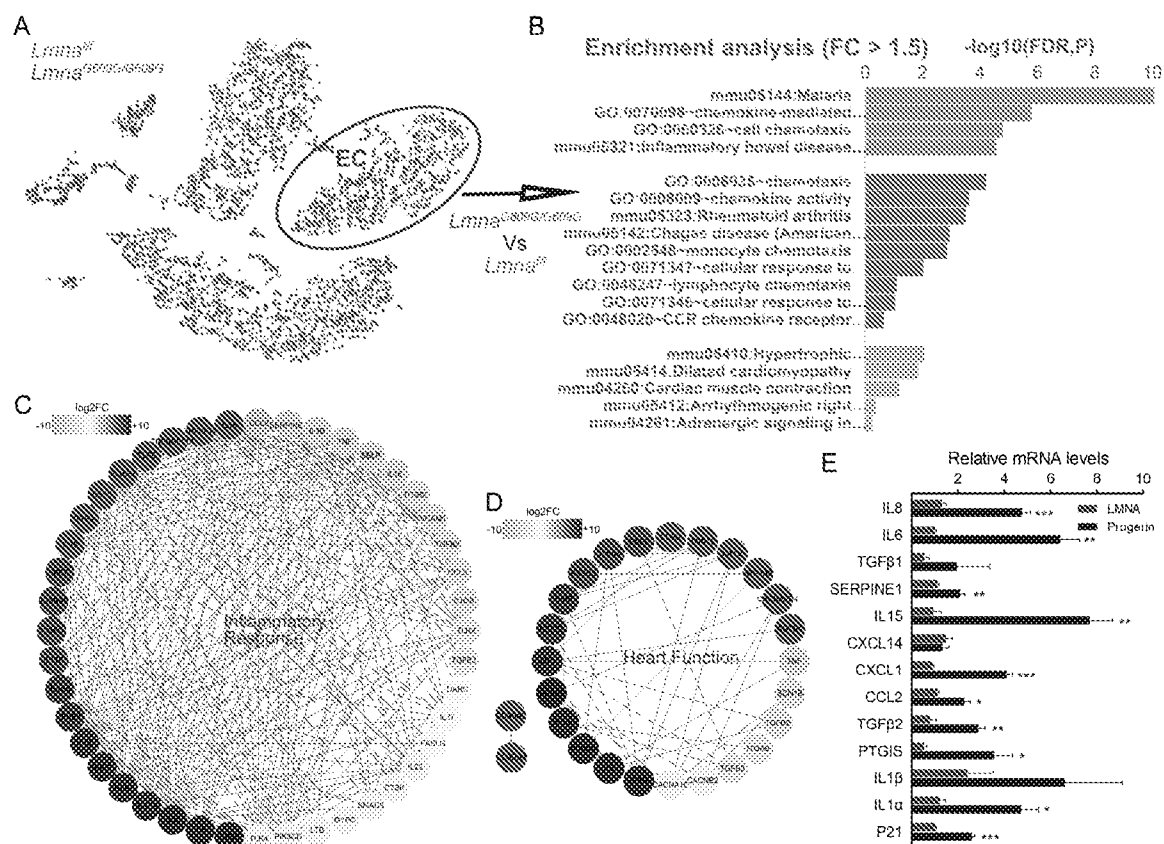


Figure 1



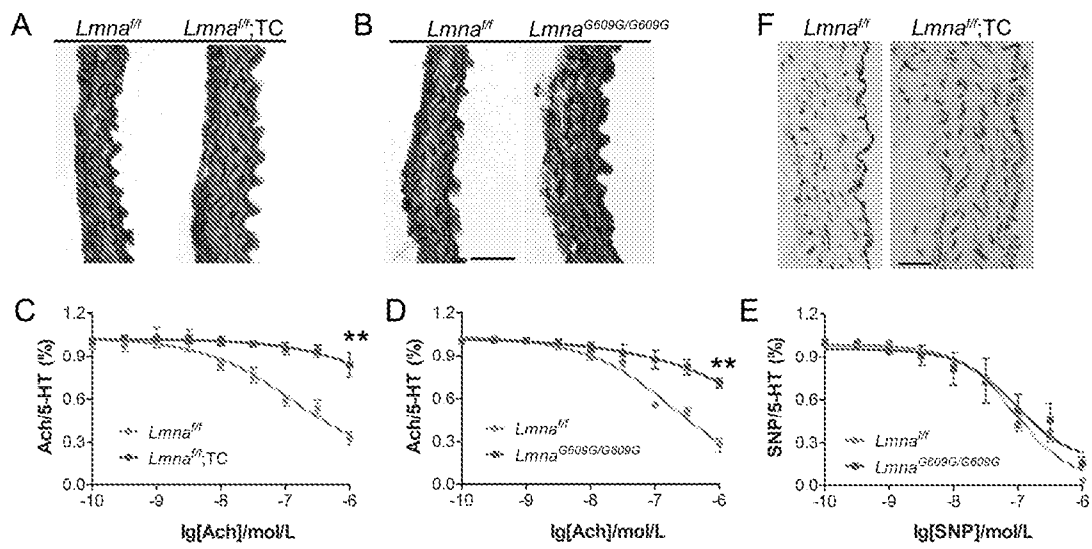


Figure 3

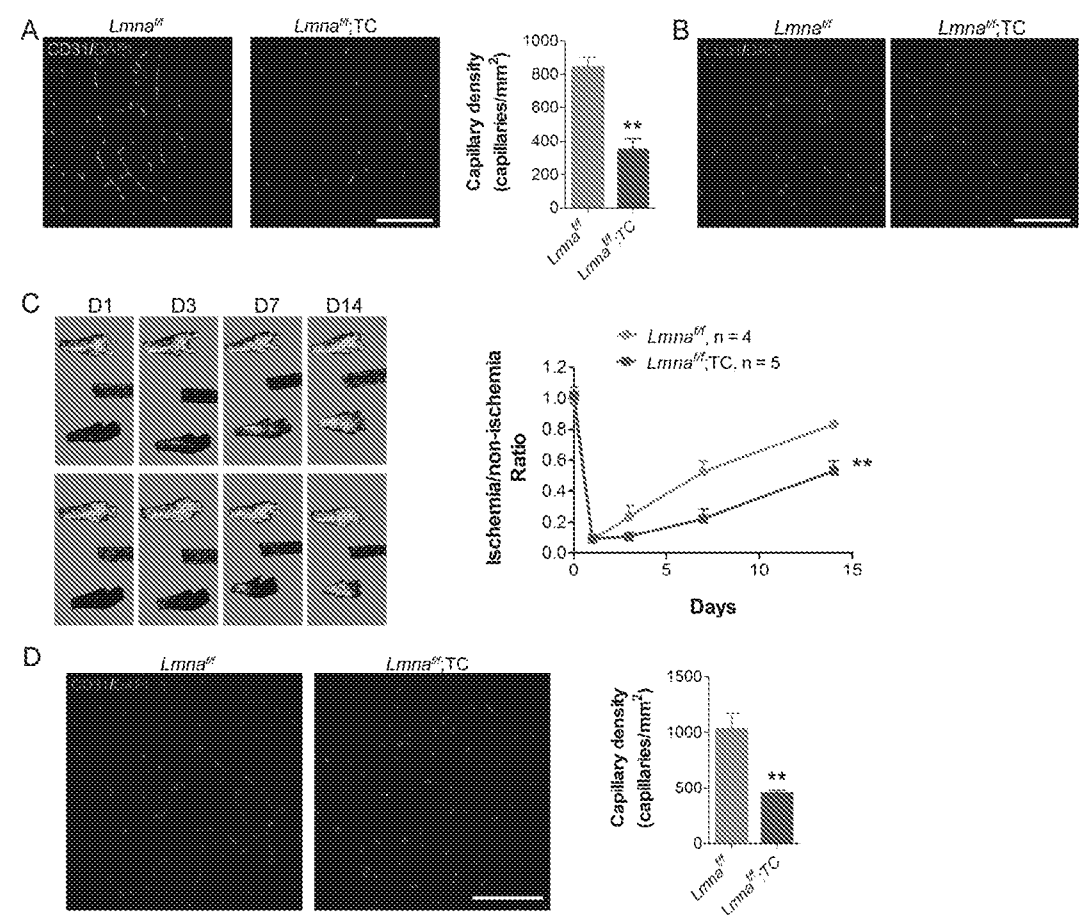


Figure 4

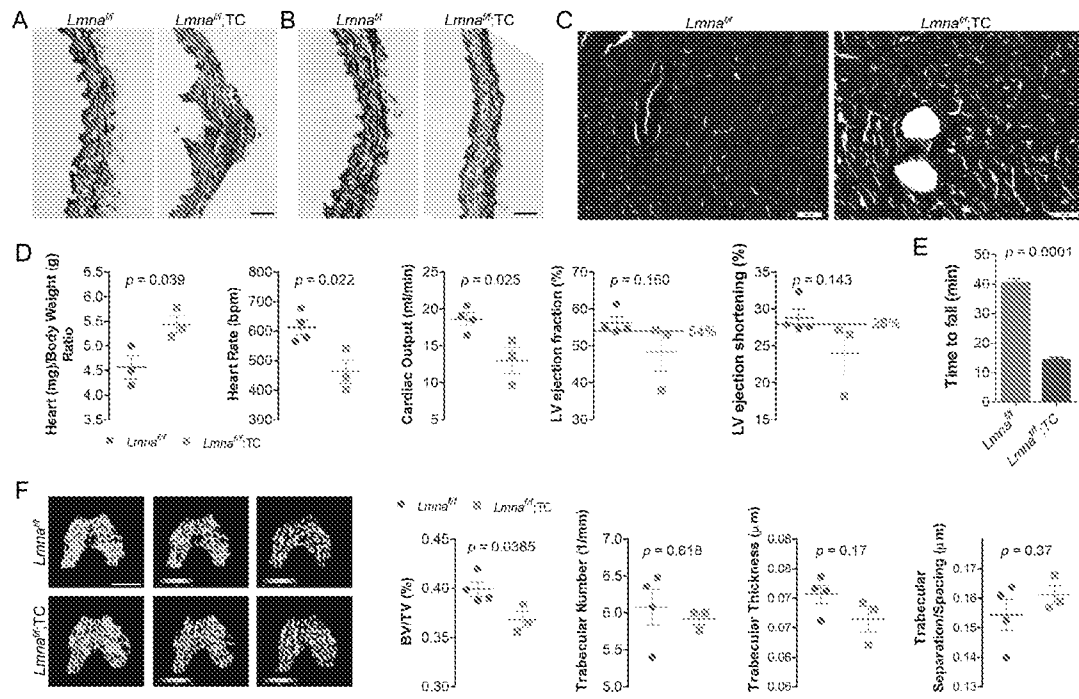


Figure 5

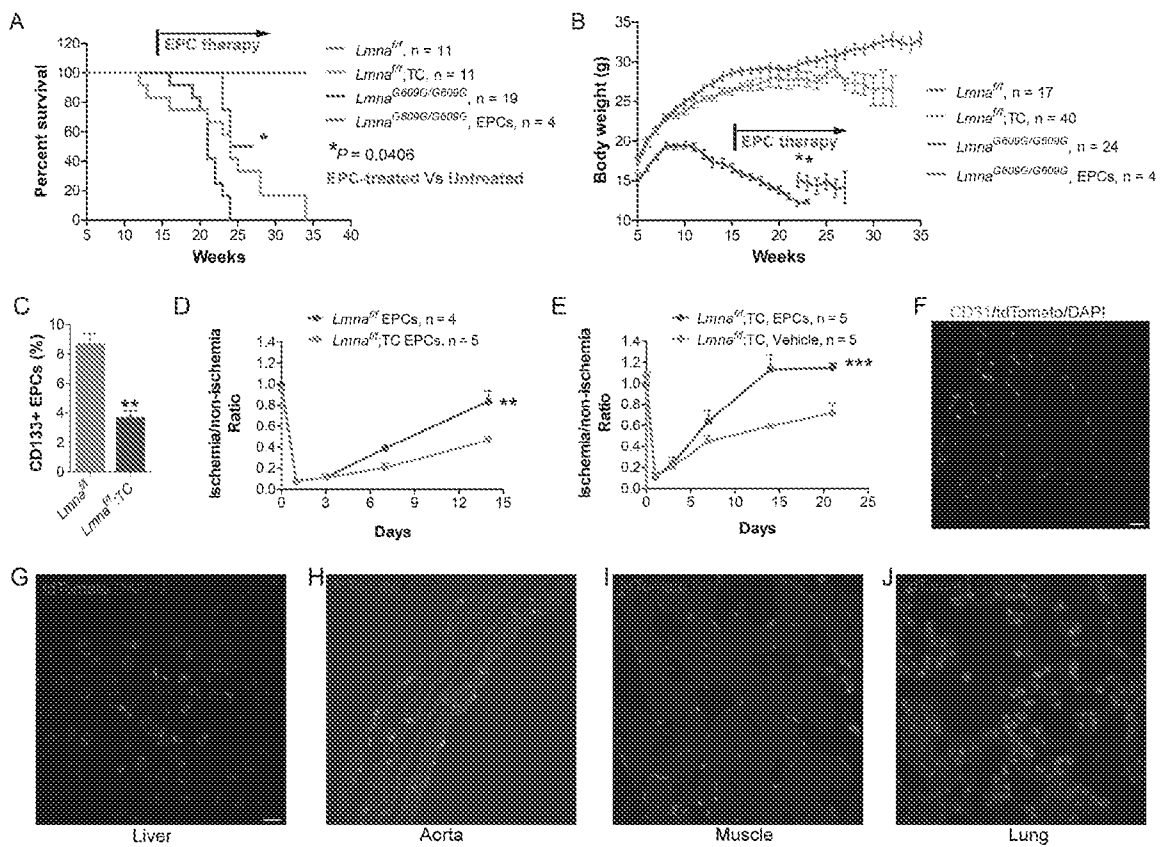


Figure 6

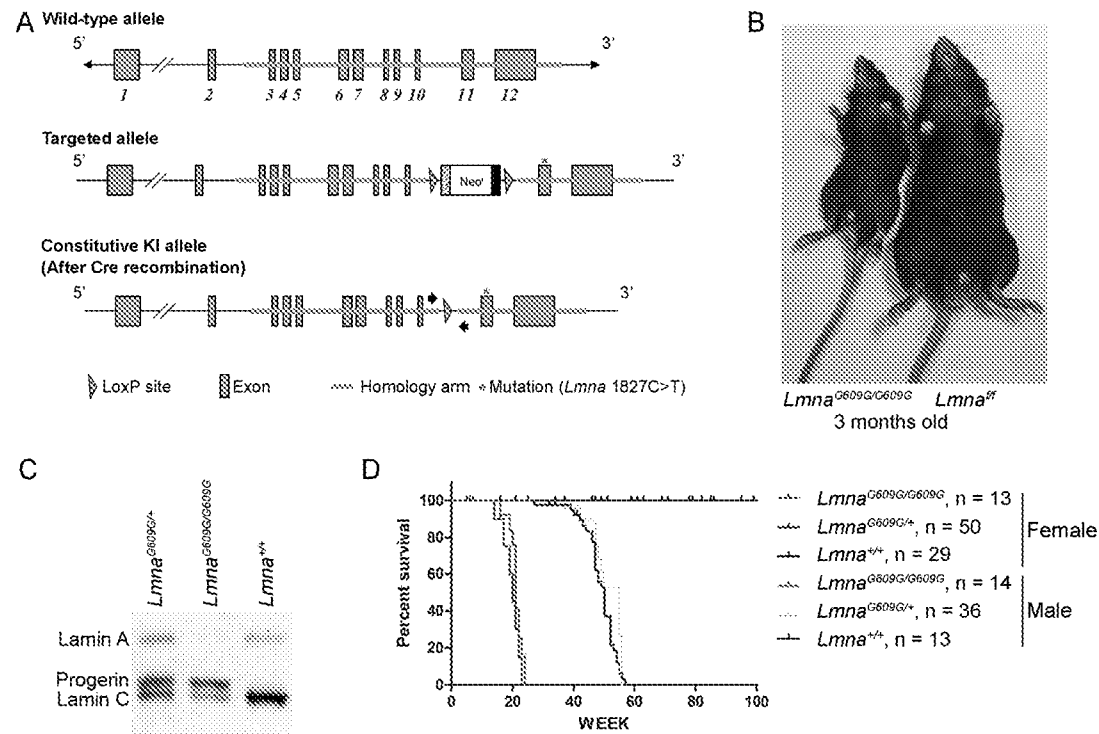


Figure 7

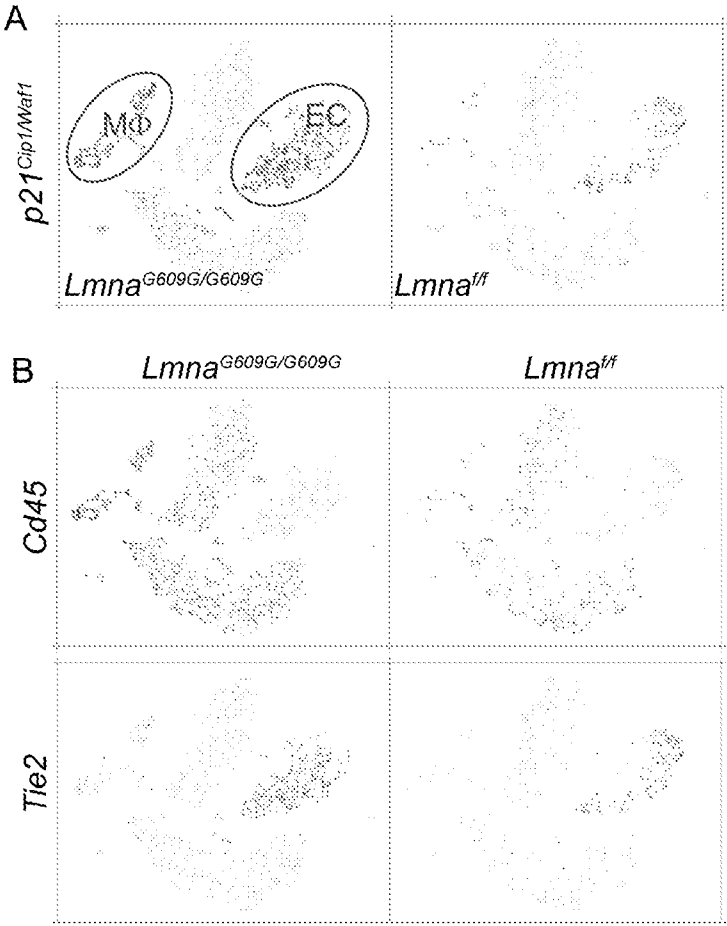


Figure 8

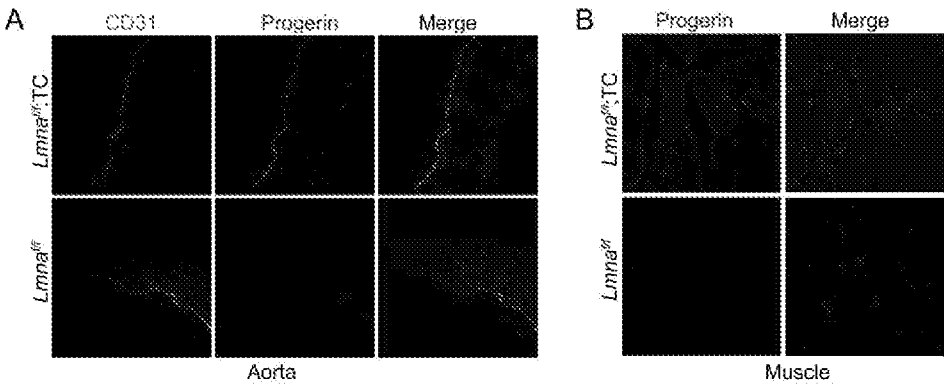


Figure 9

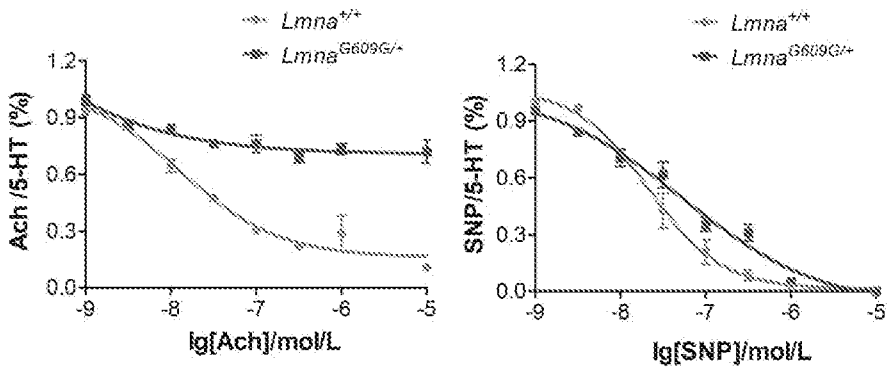


Figure 10

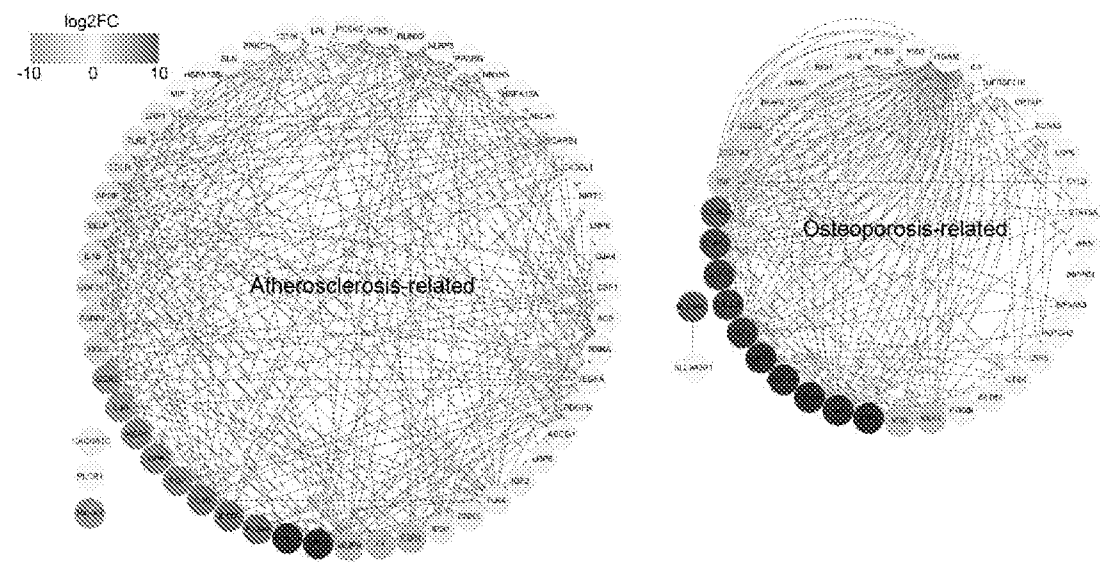


Figure 11

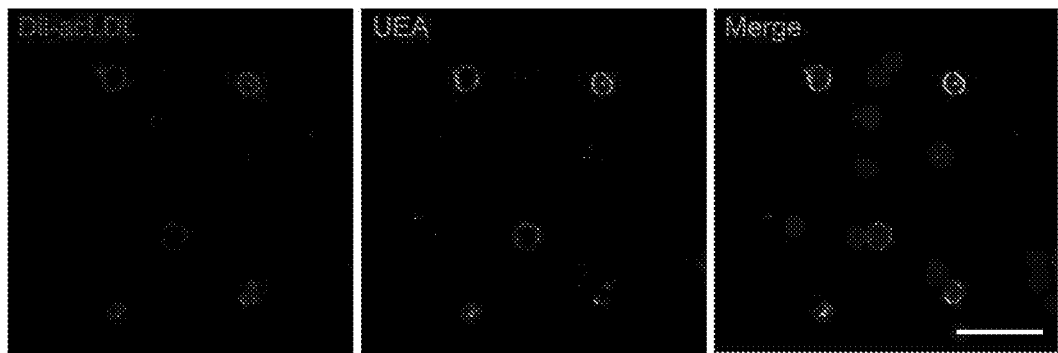


Figure 12

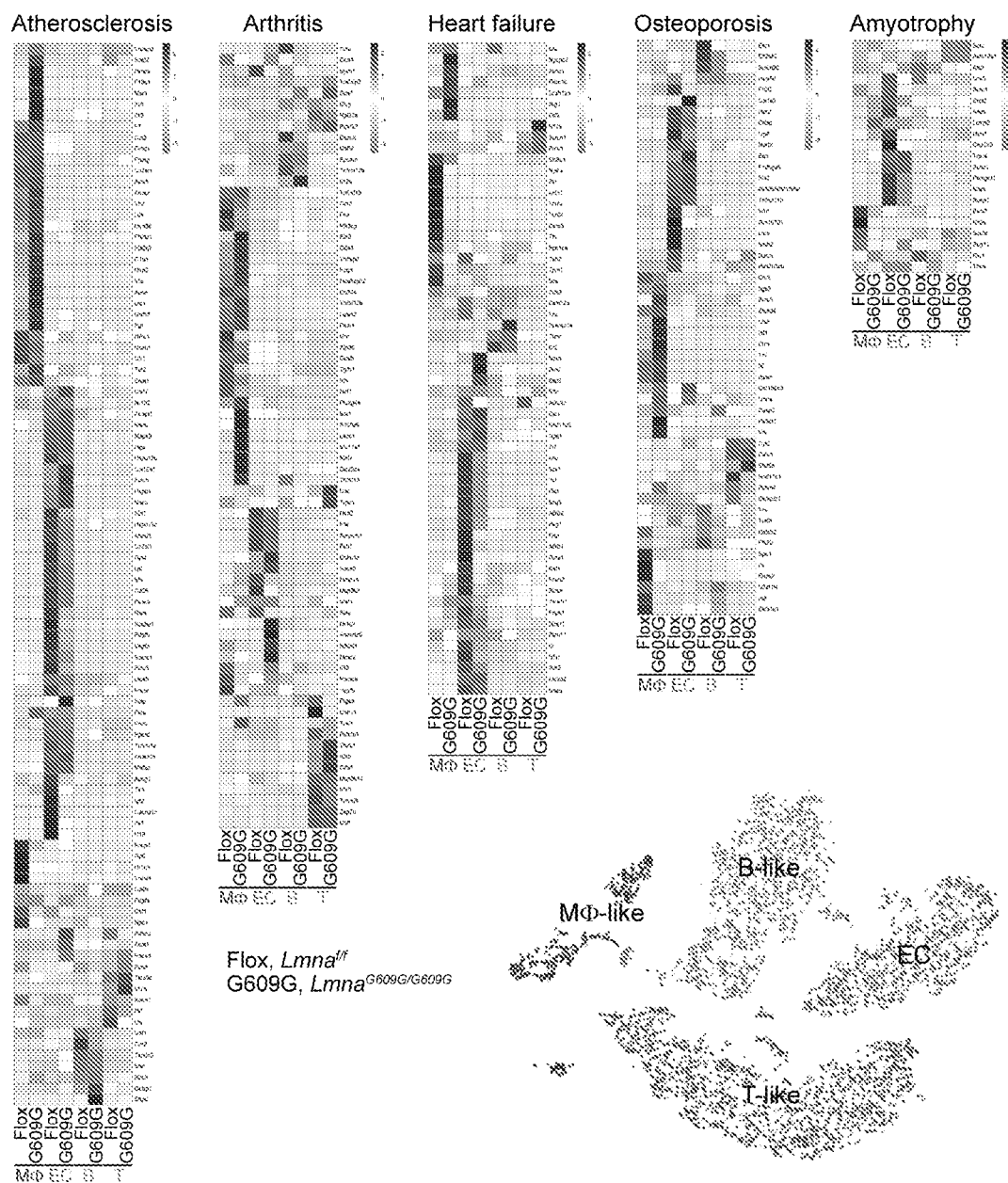


Figure 13

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2019/076488

A. CLASSIFICATION OF SUBJECT MATTER

A61K 35/44(2015.01)i; A61P 9/10(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K; A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNMED;CPRSABS;CNABS;DWPI;SIPOABS;CNTXT;WOTXT;EPTXT;JPTXT;CNKI;Pubmed;ISI web of knowledge; aging, progeria, progerin, endothelial, endothelium, EC, progenitor?, EPC, vascular, dysfunction, atherosclerosis, AS, neovascularization, CD133

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WANG X. et al. "The effects of endothelial progenitor cells on rat atherosclerosis" <i>Biotechnology and Applied Biochemistry</i> , Vol. 62, No. 2, 03 September 2014 (2014-09-03), pages 186-192 see the abstract	1-6
A	WO 2004010959 A2 (SCRIPPS RESEARCH INST) 05 February 2004 (2004-02-05) see the whole document	1-6
A	Ghebre YT et al. "Vascular Aging: Implications for Cardiovascular Disease and Therapy" <i>Translational Medicine(Sunnyvale)</i> , Vol. 6, No. 4, 30 August 2016 (2016-08-30), Article 183 see page 4, the left column, paragraph 5 - the right column, paragraph 3	1-6



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

14 November 2019

Date of mailing of the international search report

30 December 2019

Name and mailing address of the ISA/CN

National Intellectual Property Administration, PRC
6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing
100088
China

Facsimile No. (86-10)62019451

Authorized officer

LI,Kangqi

Telephone No. 62411034

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2019/076488

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **7-10**
because they relate to subject matter not required to be searched by this Authority, namely:
[1] Claims 7-10 are directed to a method of treatment of the human/animal body and thus excluded from international search according to Rule 39.1(iv).
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2019/076488

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
WO	2004010959	A2	05 February 2004	CN	1307300	C	28 March 2007
				WO	2004098499	A2	18 November 2004
				MX	PA05000972	A	12 September 2005
				CA	2493122	C	27 November 2012
				WO	2004098499	A3	06 October 2005
				BR	PI0312920	A	10 July 2007
				RU	2331669	C2	20 August 2008
				JP	5385219	B2	08 January 2014
				PL	216193	B1	31 March 2014
				JP	2005538742	A	22 December 2005
				PT	1542536	E	14 February 2012
				EP	1542536	A2	22 June 2005
				AU	2003259687	A1	16 February 2004
				US	2004197902	A1	07 October 2004
				KR	20110102961	A	19 September 2011
				CN	1688196	A	26 October 2005
				EP	1542536	A4	16 May 2007
				ES	2376866	T3	20 March 2012
				KR	20050027132	A	17 March 2005
				JP	2010285434	A	24 December 2010
				ZA	200500682	B	26 July 2006
				US	7153501	B2	26 December 2006
				AT	536405	T	15 December 2011
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				WO	2004010959	A3	07 April 2005
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